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USE OF SEPHADEX FOR RAPID PURIFICATION OF FLUORESCENCE STAINED ANTIBODY SOLUTIONS

/Following is a translation of an article by H. Wagner of the Institute for Microbiology and Experimental Therapy (Director: Prof. Dr. Habil. Gerhard Domagk) of the German Academy of Sciences, Berlin, (Department of Medical Microbiology) which appeared in the German-language periodical Zentralblatt für Bakteriologie (Bulletin of Bacteriology), Vol 185, pages 124-128, 1962./

The method of staining antibodies which was developed in 1941 by Coons and associates has found a very wide application during the past decade in immune chemistry, virology and bacteriology. However, the time-consuming and expensive process necessary for the production of the antibody staining preparation was a hindrance to a routine use of the method for rapid diagnosis of clinically important bacteria. Recent years have seen many attempts to simplify the staining of antibodies (Goldman and Carver 1957, Marshall et al, 1958). In 1960, Rinderknecht succeeded in reducing the time necessary for staining serum to 5 minutes through adsorption of the fluorescence dyes onto kieselguhr. The ensuing separation of the unbound dye by means of dialysis requiring several days remains time-consuming and unhandy, however. We have sought to perform this operation in a short period of time also. A possibility for this was offered by the method developed by Porath and Flodin in 1959 and call by them gel filtration. The method involves the use of Sephadex (a product of the Pharmacia Company of Uppsala, Sweden). With the help of this new method which resembles the column chromatography technique, high-molecular substances can easily be separated from low-molecular substances. A gel filtration in the narrower original sense is not involved, however. Based upon our experiences, this method is very suitable for the rapid purification of unbound dyes from fluorescing serums.

Methodology

A weighed amount of dry Sephadex G 50 is mixed with 0.15 n phosphate table salt buffer p_H 7.0 to a thin solution and carefully filled into a chromatography tube onto the lower end of which a G-3-sinter plate is fused. The uniform packing of the gel grains is important for a good separation. The amount of Sephadex required depends upon the volume of the serum conjugate to be cleaned. In order to obtain a complete isolation of the protein fraction from the free dye, the volume of the trial solution must be less than the volume of water retained by the gel (this value is given as water regain for every charge by the manufacturer). For 10 ml of serum, therefore, 2-3 grams of Sephadex suffice. The trial solution is placed upon the surface of the prepacked column with a pipette and as soon as it has sunked in it is followed by a further phosphate table salt buffer. It is important to insure that the surface of the column is never allowed to dry. The separation of the serum conjugate from unbound dye becomes visible after a short time (Illustration 1). The very fast-migrating conjugate reaches the end of the column in a few minutes and can be captured again there without loss due to the fact that the liquid movement can be observed readily. The slowly-migrating dye is eluted with additional amounts of buffer solution. Immediately thereafter, the column is available for further separation. It can be used for many separations without changing its characteristics.

As a means of checking whether the serum conjugate had been completely freed of dye, the eluted fractions of the column of 2 grams of Sephadex G 50 were added to 1 ml of a protein solution labelled with fluorescein isothiocyanate according to Rinderknecht and then examined spectrophotometrically (Illustration 2). The protein concentration was measured at 540 millimicrons after staining with the biuret-reagent of Weichselbaum, while the concentration of fluorescine isothio cyanate was determined directly in the trial solution through its absorption at 420 millimicron (Goldwasser and Shepard, 1958). From the experiments, there results:

1. The unbound dye is completely separated from the stained protein. The results are as good as those obtained with an extensive dialysis which requires several days.

2. The serum conjugate is eluted in a narrowly limited fraction. Its concentration is therefore only slightly decreased in the purification process. It should be mentioned here that the original protein concentration can be reconstituted with the help of the same product (Sephadex G 25 is best, but G 50 is also suitable) (Flodin et al. 1960). Serum with titers which are too low can likewise be concentrated without necessitating a precipitation of the globulins. For this, an amount of dry

Sephadex swelled according to its water-absorption ability is added to the solution. When the concentrated protein solution is removed from the swelled gel with a suction filter. Salt concentrations and p_H values remain quite constant during this process.

3. The purification of several milliliters of fluorescence stained serum is completed in about 10 minutes. It can therefore be carried out at room temperature without fear of denaturization of the albumens. This renders possible the staining of antibodies in laboratories where a sufficient refrigerated room for dialysis is not available.

Altogether, a possibility is provided for purification of fluorescence stained antibody solutions in a short time and without complication. In the production of large amounts of conjugate, the considerable saving in buffer solution might also be of importance. In connection with the staining techniques of Rinderknecht (1960), there results a method of producing a fluorescence stained and purified antibody preparation within less than one hour.

Summary:

Quantitative separation of the unbound fluorescence dye from antibody solutions labelled with fluorescence is achieved by the use of gel filtration with Sephadex G 50. As compared with the previously used dialysis, the purification is terminated in a few minutes and therefore can be done at room temperature. Due to the good possibility of optical control, it permits a regain without loss of the serum conjugate with only negligible decrease of concentration.

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Illustration 1. A Sephadex G 50 column shortly after placement of the trial solution. A = serum conjugate, B = unbound dye.

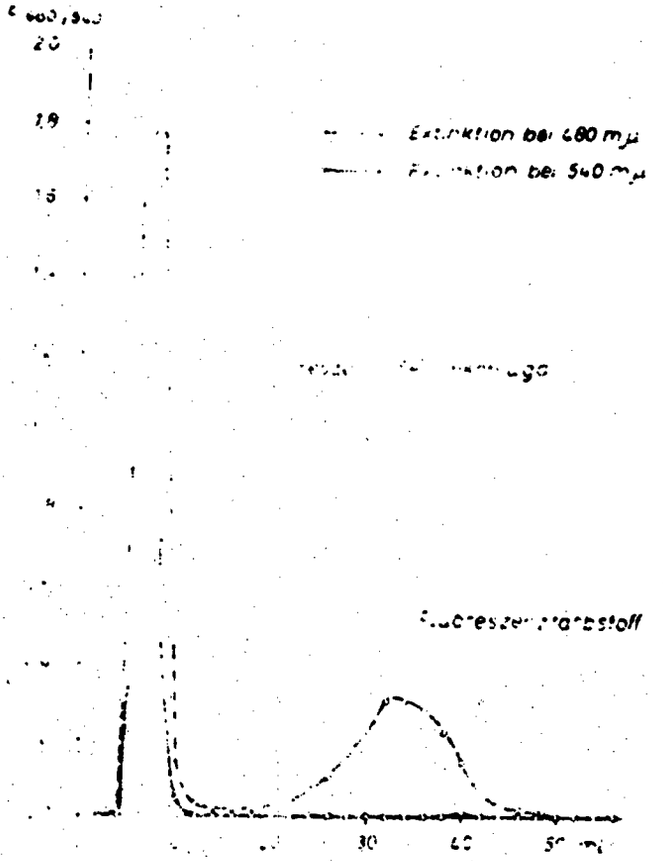

Illustration 2. Separation of the fluorescing serum from unbound fluorescoine isothio cyanate through gel filtration.

Legend: Extinktion = extinction or absorbance
Fluoreszenzfarbstoffe = fluorescence stain